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Benzoylalanine-Derived Ketoamides Carrying Vinylbenzyl Amino Residues: Discovery of Potent Water-Soluble Calpain Inhibitors with Oral Bioavailability

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Novel benzoylalanine-derived ketoamides were prepared and evaluated for calpain I inhibition. Derivatives carrying vinylbenzyl amino residues in the P_2-P_3 region inhibited calpain in nanomolar concentrations and thus represent a novel class of nonpeptidic calpain inhibitors. Selected examples exhibited an improved pharmacokinetic profile including improved water-solubility and metabolic stability. In particular, these calpain inhibitors showed oral bioavailability in rats as demonstrated by *N*-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[*E*-2-(4-diethylaminomethylphenyl)ethen-1-yl]benzamide (**5d**). The closely related derivative *N*-(1-carbamoyl-1-oxohex-1-yl)-2-[*E*-2-(4-dimethylaminomethylphenyl)-ethen-1-yl]benzamide (**5b**) was evaluated for neuroprotective efficacy after experimental traumatic brain injury in a fluid percussion model in rats. When administered after injury, **5b** reduced the number of damaged neurons by 41%, and this result would be in line with the suggested neuroprotective efficacy of calpain inhibition.

Mammalian calpains represent a family of nonlysosomal cysteine proteases of which 14 members have been reported so far.[1] The most familiar members of this family, calpain I and calpain II, have been well-known for many years.[2] More recently discovered calpains have attracted particular interest since their physiological functions have been attributed to defined human diseases. For example, calpain 10 has been postulated as a susceptibility gene for type-2 diabetes,[3] and, furthermore, the loss of activity of calpain 3 has been related to limb-girdle muscular dystrophy 2A.[4]

Calpain I is activated by calcium ions and is also called μ-calpain, according to micromolar calcium concentrations which are required for its activation in vitro. Calpain I is an intracellular protease that is ubiquitously found in man. Its physiological role is not yet fully understood but many intracellular processes such as the degradation of cytoskeleton proteins have been attributed to calpain I.[5] Over the past decade a number of more specific roles of calpain I have been described which suggest the involvement of calpain I in modulating cellular signal transduction,[6] apoptotic processes,[7] activation of platelet proteins,[8] and degradation of cyclin-dependent kinase cdck5 specific activator p35.[9] Excessive activation of calpain I triggers serious cell damage or even cell death,[10] and calpain I is presumed to contribute to the progress of a number of diseases.[11] Indeed, the inhibition of calpain I has revealed beneficial effects in experimental models of, for example, stroke,[12] myocardial infarction,[13] brain trauma,[14] and multiple sclerosis.[15] Therefore calpain I has attracted interest in drug discovery research.[16] But, though substantial efforts were focused on calpain inhibitors for many years, up to now, no clinical trial investigating the therapeutic efficacy of calpain inhibitors in human disease has been reported.[17]

Most of the known calpain inhibitors bind to the catalytic center in a competitive manner and are derived from small peptides which are structurally related to the cleavage site of calpain substrates.[18] Irreversible calpain inhibitors are designed by assembling a backbone and a particular reactive moiety capable of entering an irreversible covalent bond with the cysteine present in the catalytic center. Well-known irreversible reactive moiety are epoxides, diazoketones, α-haloketones, and vinyl sulfones. Most reversible calpain inhibitors represent transition state mimetics consisting of a backbone and an aldehyde or a ketone as the reactive moiety which enter a reversible covalent bond with the cysteine residue in the catalytic center. Two of the well-known reversible inhibitors, MDL 28170 (**1**)[19] and AK 295 (**2**),[20] have the dipeptides Z-Val-Phe and Z-Leu-Abu, respectively, as backbones and either an aldehyde or a ketoamide moiety as a reactive moiety (Figure 1). Some progress has been made in modifying the peptidic backbone, and nonpeptidic calpain inhibitors have been discovered.[17,21] In general, using such aldehyde and ketone moieties as reactive moiety can give rise to problems with respect to stability, metabolism, and selectivity. Accordingly, the peptidic aldehyde MDL 28170 (**1**) shows typical structure-related disadvantages, such as decomposition during storage and excessive metabolism.[22] The ketoamide AK 295 (**2**), which is one of the first water-soluble, reversible calpain inhibitors, is only effective when administered via a special route.[20] Efforts to discover an appropriate surrogate for the highly reactive aldehyde or ketone as reactive moiety have been unsuccessful. Other functional groups were examined as reactive moiety, but most failed due to their modest inhibition of calpain.[23,29] Moreover, there have been few reports of calpain inhibi-

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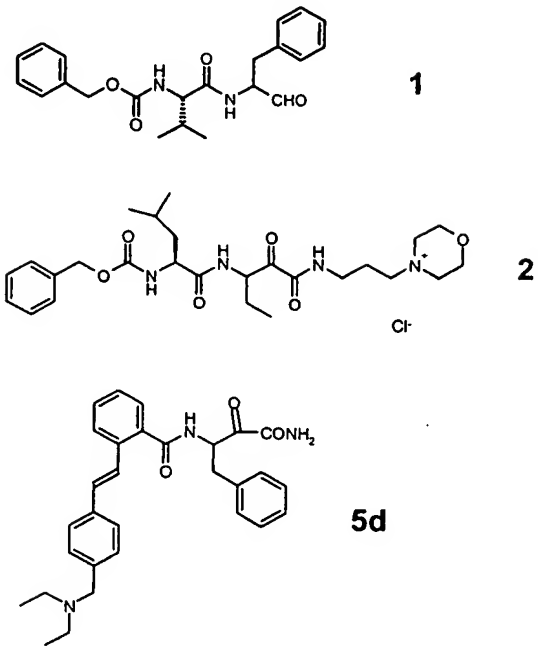

Figure 1.

tors exhibiting the oral activity which is required for them to be used in some of the envisaged therapies.[24] Hence, the use of the calpain inhibitors, so far reported, is limited for one or more of the following reasons: poor selectivity, poor metabolic stability, low cellular penetration, poor kinetics, and, depending on the envisaged therapeutic indication, low oral availability and low water-solubility.[17]

Our approach was to design novel calpain inhibitors, which had fewer peptidelike features in order to obtain more stable structures. First, we focused our efforts on discovering novel nonpeptidic backbones appropriate for reversible calpain inhibitors. Previously, we reported substituted naphthoyl piperidines derived from ketoamides as reactive moiety to be potent calpain inhibitors.[25] Proceeding these efforts we discovered the benzoylalanine derivative **3** which was a moderately potent calpain inhibitor (Figure 2). Optimization of the substitution pattern at the benzoyl moiety, in particular in the ortho-position, has enabled us to design potent calpain inhibitors such as **4**.[26] Interestingly, we noticed that several ketoamide-derived inhibitors within this set had improved metabolic stability in vitro and in vivo. Encouraged by this finding we focused our efforts on derivative **5** carrying a styryl residue at the benzoyl moiety and a ketoamide as reactive moiety which, ultimately, led us to the discovery of aminomethyl derivatives such as **5d**, which exhibit superior properties such as water-solubility, potent calpain inhibition, improved metabolic stability, and oral bioavailability.

The syntheses of the novel benzoylalanine-derived ketoamides are outlined in Figures 3 and 4.

The P_1 building block 3-amino-2-hydroxyheptanoic acid amide (**11a**) was available starting from 1-nitropentane (**6**) (Figure 3). Compound **6** was added to glyoxalic acid at ambient temperature to give a diastereomeric mixture of the nitro alcohol **8** in over 90% yield. Hydrogenation of the crude **8** with Pd/C at an elevated temperature (50 °C) gave the aminocarboxylate **9**. The amino group in **9** was protected using $(Boc)_2O$

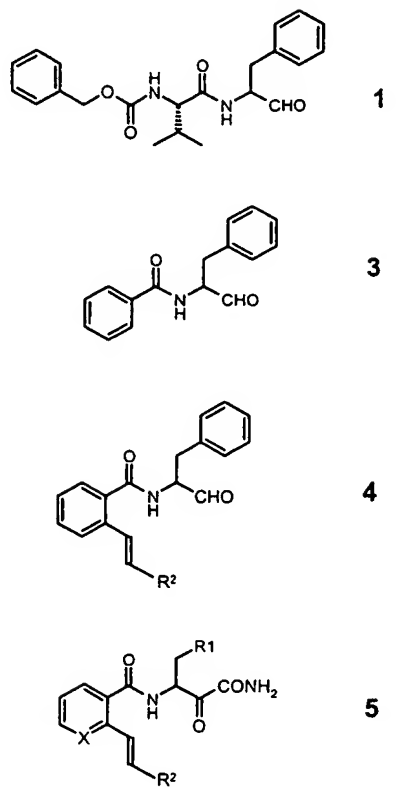

Figure 2.

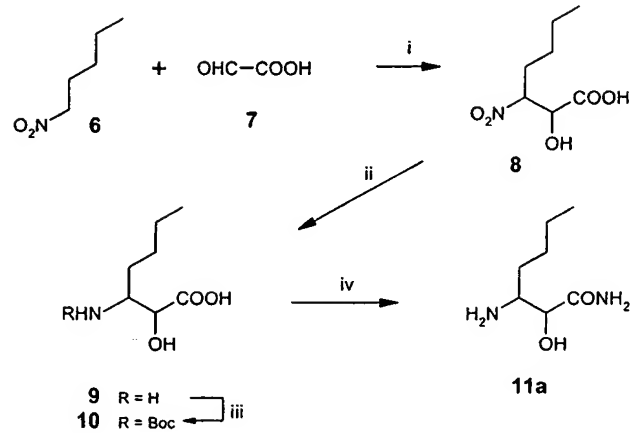

Figure 3. Route of synthesis for the P_1 building block **11a**. (i) CH_3OH, Et_3N, rt.; (ii) AcOH, H_2, Pd/C, 50 °C; (iii) CH_3OH, $(Boc)_2O$, rt.; (iv) 1. THF/DMF, NH_3, EDC, HOBt, rt.; 2. HCl.

and subsequently coupled to NH_3 using the EDC/HOBt coupling variant. After removal of the protection group and recrystallization of the resulting amine, a single diastereomer of amine **11a** was obtained. The corresponding phenyl derivative **11b** was prepared according Harbenson et al.[27]

The stilbene carboxylates **14** were prepared via Heck reactions. Styrols **13** were added to *o*-bromobenzoic carboxylate **12** in the presence of $Pd[(o\text{-}tol_3P)_2Cl]_2$ at 100 °C to give the esters **14** in 50–100% yields. However, the corresponding pyridine derivative **14c** was only obtained in lower yield (42%), which may be due to the use of an aromatic chloride **12b**. *E*-Configured stilbenes **14** were formed predominately in these Heck reactions. The *E*-configuration of these stilbene derivatives was determined according to the coupling constants (J=15–17 Hz) between the two olefinic protons. In a final step,

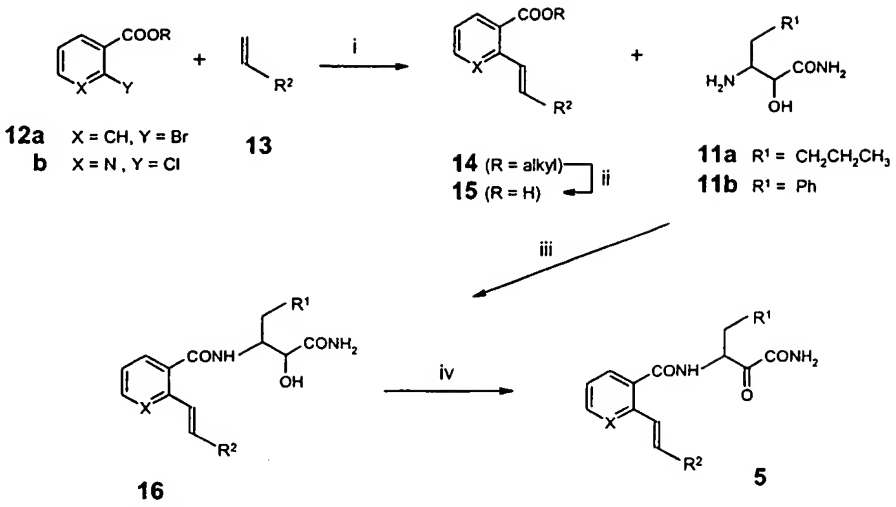

Figure 4. Route of synthesis for the envisaged calpain inhibitors **5**. (i) DMF, $Pd(OAc)_2$, $(o\text{-}Tol)_3P$, 100 °C; (ii) THF/H_2O, KOH; (iii) DMF/CH_2Cl_2, EDC, HOBt, (iv) DMSO, DCC.

Table 1. Synthesized Compounds and Their Results in Inhibition of Human Calpain I (K_i) and in Inhibition of the Degradation of the Tyrosine Kinase Pp60src (IC_{50}) in Human Platelets

	X	R^1	R^2	calpain I K_i (nM)[b]	cathepsin B K_i (nM)[b]	pp60src IC_{50} (μM)[c]
3	-	-	H	1080[26]	nt[a]	nt[a]
4a	-	-	CH=CH−Ph	140[26]	nt[a]	nt[a]
4b	-	-	2-naphthyl	15[26]	nt[a]	nt[a]
5a	CH	phenyl	2-naphthyl	8.4 ± 2.7	99 ± 15	1.4
5b	CH	$CH_2CH_2CH_3$	4-(C_6H_4)-$CH_2N(CH_3)_2$	13.3 ± 1.5	27 ± 6	0.8
5c	N	phenyl	4-(C_6H_4)-$CH_2N(CH_3)_2$	18.3 ± 1.2	83 ± 11	1.0
5d	CH	phenyl	4-(C_6H_4)-$CH_2N(CH_2CH_3)_2$	27.0 ± 2.5	62 ± 9	0.7
MDL 28170	-	-	-	15.5 ± 2.0	18 ± 3	0.7

[a] Not tested. [b] Mean ± SEM. [c] Mean of two or more independent experiments.

all esters **14** were hydrolyzed with diluted NaOH or KOH to afford the carboxylates **15.** These carboxylates **15** were coupled to the amines **11** by the EDC/HOBt coupling variant to give the amides **16**. These amides **16**, all of which carry an secondary alcohol group, were oxidized by DMSO/DCC at ambient temperature which resulted in the envisaged ketoamides **5**. Compound **5d** was converted into a methanesulfonate because of its excellent solubility in water (>5%) whereas both **5b** and **5c** were used as hydrochlorides.

The prepared ketoamides **5** were evaluated for inhibition of calpain I in a common assay using human calpain I isolated from erythrocytes and Suc-Leu-Tyr-AMC as the fluorogenic substrate.[28] The results are summarized in Table 1.

All derivatives **5** carried a ketoamide as reactive moiety and displayed a reversible mode of action (data not shown). These four compounds were potent calpain inhibitors, which confirmed the ketoamide moiety to be eminently useful as a warhead in calpain inhibitor design. The naphthalene **5a** was the most potent inhibitor within the present set and had a K_i of 8.4 nM. Compounds **5b**, **5c**, and **5d** displayed slightly diminished potency in calpain I inhibition. Their K_is were 13.3 nM, 18.3 nM, and 27.0 nM, respectively.

Our approach used the benzoyl derivative **3** as the starting point carrying an aldehyde warhead. Derivate **3** showed only modest affinity for calpain I (K_i = 1.08 μM).[29] The insertion of a phenylvinyl or naphthylvinyl moiety in the ortho-position of this benzoyl residue resulted in 9-fold and 70-fold higher potency, respectively. Interestingly, within this structural class both aldehydes and ketoamides as reactive moiety exhibited similar potency for calpain inhibition. For instance, the naphthylvinyl ketoamide **5a** displayed equally high potency in calpain inhibition as the corresponding aldehyde.[26] A typical feature of these inhibitors may be the ortho-substitution at the benzoyl moiety. The favorable long bulky residues attached to this benzoyl moiety suggest a hydrophobic cave in the P_2-P_3 region at the enzyme-binding pocket in which these residues fit well. The size of this pocket might be huge since the large distal vinyl naphthalene residue seemed favorable for affinity. This is in agreement with other studies demonstrating favorable effect on affinity by large lipohilic residues in P_2-P_3.[30] Most of the reported transition-state mimetic calpain inhibitors carry polar groups such as an amide or a sulfonamide within the P_2-P_3 region, which are considered to be just as strongly favorable in calpain inhibition.[21] Surprisingly, the above-mentioned derivatives such as **5a** do not carry any polar moieties within this region, which indicates that the incorporation of an optimized lipophilic side chain in P_2-P_3 overcomes the lack of such an important polar ligand-enzyme interaction.

Insertion of aminomethyl moiety into the para-position at the phenylvinyl residue in **4** also resulted in calpain inhibitors which were 5- to 10-fold superior in potency. This finding appears to be contradictory to the above results since both the insertion of lipophilic residues, such as the annellated benzene ring (**5a**), and a highly polar residue, such as a basic amino group (**5b–d**), as substituents at the distal phenylvinyl moiety was favorable. It is unclear whether the amines **5b–d** fit in

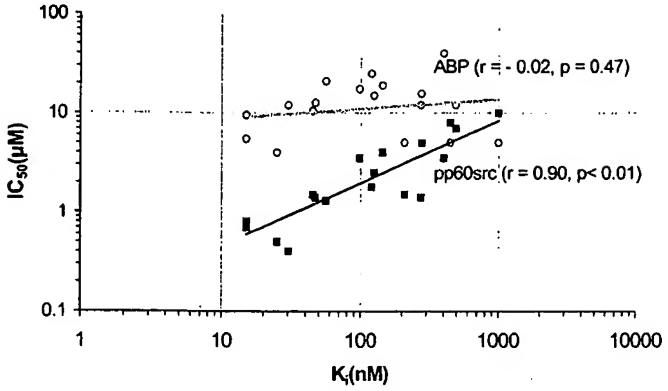

Figure 5. Correlation of inhibition constants K_i of calpain I inhibition and either actin-binding protein (ABP) degradation (IC_{50}, open circles, gray line) or pp60src degradation (IC_{50}, squares, black line) of a set of calpain inhibitors in the human platelet assay. The set includes AK295, MDL 28170, the herein presented inhibitors, and a number of corresponding derivatives (data not shown). A linear regression analyses was used to determine the correlation coefficient r.

this enzyme-binding pocket in the same manner as the naphthalene **5a**. Further studies with the recently reported X-ray structure of μ-calpain might help to elucidate these findings.[31,32]

Since calpain I is an intracellular enzyme, the capability of inhibitors to cross cell membranes is of considerable importance. In general, the penetration of calpain inhibitors into cells has been estimated by their inhibition of calpain-mediated intracellular protein degradation. Several cytoskeletal proteins, such as spectrin, MAP2, actin-binding protein (ABP), talin etc., were evaluated, and extracellular applicated calpain inhibitors attenuated or blocked their degradation.[32] In a first approach we examined the calpain-mediated degradation of actin binding protein (ABP) and talin in human platelets.[33] A number of peptidic and nonpeptidic calpain inhibitors were evaluated and, indeed, they inhibited the formation of the corresponding degradation products (data not shown). However, most compounds exhibited rather similar cellular efficacy (expressed as IC_{50}), raising questions on the correlation of these efficacies with calpain inhibition. We examined the correlation of the cellular activity (IC_{50}) and calpain inhibition (K_i) by a linear regression analysis and, in fact, observed no correlation ($r = -0.02$, $p = 0.47$, Figure 5). Of course, the differential ability of the compounds to penetrate into cells might impede any correlation. However even structurally very closely related derivatives displayed similar potencies in this assay inspite of substantial differences in their K_i values for calpain inhibition. Interestingly, similar small variations in the cellular efficacy of calpain inhibitors were reported when using another cytoskeleton protein, spectrin, as a marker for calpain activity.[34] To overcome this problem we searched for other calpain degraded proteins and selected the tyrosine kinase pp60src which was identified as calpain substrate by Oda et al.[35] In an adapted assay, calpain in human platelets was activated by adding the calcium-ionophore A23187 and calcium, and the pp60src degradation products with molecular weights of 52kDa and 47kDa were identified by Western blotting. The compounds exhibited concentration-dependent inhibition of the pp60src degradation, which enabled us to determine

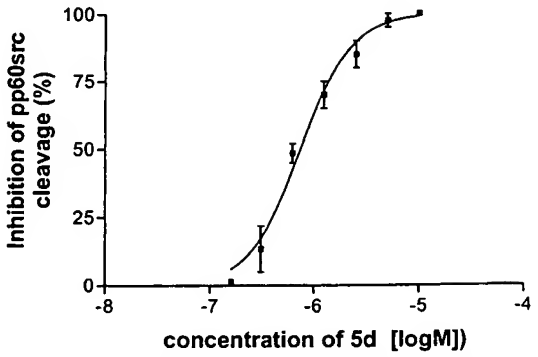

Figure 6. Dose-dependent inhibition of the pp60src degradation in human platelets by calpain inhibitor **5d**. Shown are percent inhibition of the cleavage of pp60src versus the inhibitor concentration.

IC_{50} values, as demonstrated by **5d** (Figure 6). The IC_{50} values in the pp60src assay were generally 5- to 10-fold lower than in the ABP assay.

We also evaluated these results of the same set of calpain inhibitors by a linear regression analysis (Figure 5) and obtained a good correlation ($r = 0.90$, $p < 0.001$) between enzymatic calpain inhibition (K_i) and cellular inhibition of pp60src degradation (IC_{50}). Therefore this assay appeared to be useful to examine the cellular penetration of calpain inhibitors.

The calpain inhibitors **5a–d** were evaluated in this pp60src-assay and exhibited IC_{50}s ranging from 0.6 and 1.4 μM, which demonstrates that all four inhibitors penetrated into cells (Table 1), even though there was a more than 30-fold decrease in cellular activity (IC_{50}) compared to enzymatic inhibition (K_i). This decrease is in line with the results reported elsewere by other groups using spectrin as a marker.[34] At first glance, these differences in activity may indicate poor or moderate ability of the inhibitors to penetrate into cells. But the inhibitors were present within cells immediately and were equipotent when the time of incubation was varied (data not shown). Unspecific intracellular protein binding of the inhibitors may have impact on their free concentration and therefore on the IC_{50}s in this assay. However, the inhibitors **5b–d** showed protein binding below 90% which are acceptable values ruling out significant problems due to high protein binding (data not shown). Both the good correlation of the pp60src assay and the similar magnitude of declines of cellular potency suggest the involvement of other unknown factors in these cellular calpain assays. It is unclear whether these declines in cellular activity may have impact on the results in vivo.

We also evaluated whether the novel calpain **5a–d** inhibitors block other cysteine proteases such as cathepsin B, papain, and caspase 3 (data not shown). All four compounds **5a–d** inhibit cathepsin B with K_is ranging from 20 to 100 nM. No inhibition was seen for papain and caspase 3 up to 10 μM. Therefore these inhibitors exhibit a similar profile like many other reported transition state mimetic calpain inhibitors blocking both calpain and cathepsin B whereas good selectivity versus other cysteine proteases was observed.

One major issue in our search for novel calpain inhibitors was to improve their pharmacokinetic profile. Therefore, the novel inhibitors **5** were evaluated for

physicochemical and pharmacokinetic parameters including oral bioavailability.

With the exception of **5a**, the present inhibitors **5** showed considerable water-solubility as salts at pH 5 to 5.5 which enabled the intravenous administration of aqueous solutions. The compound **5d** as a methanesulfonate even exhibited a solubility of over 5% in water. At pH values of around 7, only **5c** displayed considerable water-solubility which may be due to its pyridine moiety.

At pH 7, the log P values of **5b**, **5c**, and **5d** were estimated to be 3.4, 2.9, and 2.8, respectively, which are fairly good values for compounds to cross cell membranes.[36]

The metabolic stability in vitro was evaluated using porcine liver subfractions.[37] After incubation of **5b**, **5c**, and **5d** in the presence of microsomes for 30 min at 37 °C, >95%, >95%, and >85% of the parent compounds, respectively, were still unchanged which indicated poor or moderate metabolic degradation by liver enzymes. Therefore these compounds were further evaluated for their pharmacokinetic profile in rats after intravenous and oral administration.[44] Representative data for **5d** may be summarized as follows. There was a linear correlation between dosages and the plasma AUCs after oral administration of the compound. Intravenous administration of **5d** gave a half-life of 1.4 h, a clearance rate CL of 1.7 L/h/kg, and a distribution volume of 3.6 L/kg. After oral administration, the timepoint of maximum concentration t_{max} was reached at 2 h, and half-life $t_{1/2}$ was 3.3 h. The clearance rate CL was 1.9 L/h/kg and oral bioavailability was determined as $F = 89\%$. Accordingly, **5d** showed an acceptable half-life and high bioavailability after oral administration. The enhanced distribution volume prompted us to evaluate tissue levels as the inhibitor concentration in the tissue and cells might be most closely related to the efficacy of calpain inhibition. Indeed, we observed considerably higher levels of **5d** in tissues such as the heart, lung, liver, and kidney, which reached 5- to 10-fold higher levels than in the plasma.

Calpain inhibitors were suggested as useful agents in the treatment of traumatic brain injury (TBI). After experimental traumatic brain injury, substantial loss of cytoskeletal proteins was observed which was attributed to enhanced calpain activity.[38] Furthermore, the calpain inhibitor AK 295 (**2**) attenuated motor and cognitive deficits following experimental brain injury in rats.[39] However, these studies gave rise to controversial data since injury-induced spectrin breakdown and cortical lesion size were not affected by AK 295. This is remarkable since a decrease in spectrin degradation is often used as a marker of calpain inhibition in vitro and in vivo.[40]

We evaluated **5b** for efficacy in experimental traumatic brain injury using the lateral fluid percussion method (FP method) in rats. The lateral fluid percussion method was developed to create a model of traumatic brain injury in the rat for the evaluation of potential neuroprotective compounds.[41] This technique induces a controlled and consistent injury to tissue underlying the site of impact, including the neocortex, hippocampus, and thalamus. Rats were subjected to brain injury by a burst of fluid pressure (2.5−2.9 bar) on the exposed

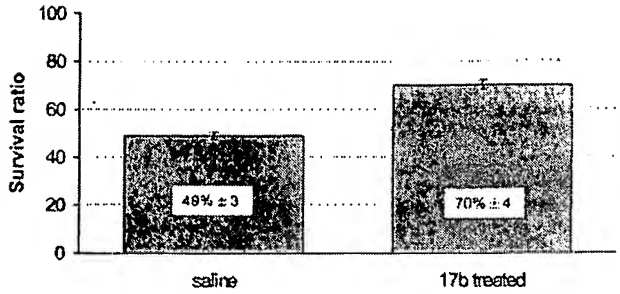

Figure 7. Efficacy of **5b** on the survival rate of neurons after experimental traumatic brain injury performed by the lateral fluid percussion method in rats. 40 mg/kg (15 min) and 20 mg/kg (120 min) of **5b** were administered ip after inducing injury. After 14 days efficacy was determined as survival ratio, which is a ratio of intact neurons of the ipsilateral side and the undamaged contralateral dentate gyrus and is expressed as mean percentage ± SEM ($p < 0.001$).

brains. Fourteen days later the animals were sacrificed and the brains removed. After Toluidine Blue staining, the intact hilus cells of the dentate gyrus were counted. For evaluation of the efficacy we examined both the ipsilateral and the contralateral side using the contralateral side as a control. The percentage of the neurons which survived (survival ratio) was calculated by the ratio of the number of the intact neurons within the ipsilateral and the contralateral side. In control animals, the survival ratio was 49% ± 3 which means that about one-half of all neurons in the dentate gyrus died within the observation period of 14 days. To evaluate the calpain inhibitor **5b**, the compound was administered intraperitoneally (ip) at 15 min (40 mg/kg) and 120 min (20 mg/kg) after inducing the lesion (Figure 7). The survival ratio of neurons in these treated animals rose to 70% ± 4 ($p < 0.001$). Therefore, the treatment by **5b** reduced the cell death in the dentate gyrus by 41%. Consequently, the calpain inhibitor **5b** demonstrated significant efficacy in protecting neurons from cell death after experimental traumatic brain injury even when administered after inducing injury and thus would be in line with the suggestion that calpain inhibition may be beneficial in the treatment of traumatic brain injury.

In summary, we envisaged superior calpain I inhibitors using benzoylalanine-derived backbones and ketoamides as warheads. Recognizing the significance of *o*-phenylvinyl residues at the benzoyl moiety we designed novel potent calpain inhibitors. The insertion of an aminomethyl residue as para-substituents at the distal phenylvinyl moiety resulted in potent calpain inhibitors **5**. All three derivatives, **5b** (A-705239), **5c** (A-558693), and **5d** (A-705253), exhibited substantially improved pharmacokinetic profiles, in particular water-solubility and metabolic stability. These inhibitors also showed oral bioavailability as demonstrated by **5d**. The derivative **5b** was evaluated for neuroprotective efficacy after experimental traumatic brain injury in the lateral fluid percussion rat model. Derivative **5b** reduced neuronal cell death by 41% and revealed significant neuroprotective efficacy even when administered after inducing the injury. This result would be in line with the proposed use of calpain inhibitors in the treatment of traumatic brain injury.[14] The evaluation of these calpain inhibitors in further pharmacological models designed

to investigate therapeutic efficacy in human diseases will be reported elsewhere.

Experimental Section

^{1}H NMR spectra were recorded either on a Varian Innova 400 MHz, a Bruker Avance 400 MHz, or a Bruker Avance 500 MHz spectrometer with tetramethylsilane as an internal standard. ^{13}C NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. A coupling constant J is given in detail when it it is necessary for determination the configuration. Electrospray ionization mass spectra (ESI) in a low-resolution mode or fast atom bombardment (FAB) mass spectra were recorded on a Finnigan Mat 90 (EI) or a Finnigan TSQ 700 (ESI) spectrometer. Elementary analyses were performed on a LECO 1000. Melting points were determined on a Büchi 530 and were uncorrected.

3-Amino-2-hydroxyheptanoic acid amide (11a): 118.5 g (1 mol) of 1-nitropentane and 204.4 g (2 mol) of Et_3N were dissolved in 400 mL of CH_3OH. To this solution was added 93.1 g (1 mol) of glyoxalic acid hydrate gradually over 2h, and afterward the reaction mixture was stirred for 16 h at ambient temperature. The solvent was removed in vacuo. The resulting residue was dissolved in H_2O and washed with ether. The pH of the aqueous phase was adjusted to 5 by adding citric acid and extracted with CH_2Cl_2. The organic phase was dried and evaporated in vacuo to yield 196 g of the crude 3-amino-2-hydroxyheptanoic acid (**8a**) as a mixture of both diastereomers. **8a** (196 g) was dissolved in 1100 mL of glacial acetic acid, and, after adding 20 g Pd/C, the reaction mixture was hydrogenated at 50°C and 1 atm. The reaction mixture was filtered, and the solvent of the filtrate was removed in vacuo. The residue was treated with 2-propanol, and the resulting precipitate was collected and dried to give 80 g of 3-amino-2-hydroxyheptanoic acid (**9a**) as a white crystalline solid. **9a** (79 g) and 200 g of Et_3N were dissolved in 500 mL of CH_3OH, and 118.5 g $(Boc)_2O$ was added. The reaction mixture was stirred for 16h at ambient temperature. The solvent was removed in vacuo. The residue was poured into 1000 mL of H_2O, and this aqueous phase was extracted with ether. The ether phase was discarded. The pH value of the aqueous phase was adjusted to 3−4 by adding 2 M HCl, and the resulting phase was extracted with CH_2Cl_2. This organic phase was dried, and the solvent was removed in vacuo to afford 139 g of the crude product 3-*tert*-butoxycarbonylamino-2-hydroxyheptanoic acid (**10a**). Crude **10a** (122.5 g) was dissolved in 700 mL of THF/DMF (6/1). HOBt (63.3 g) was added, and the reaction mixture was stirred for 15min. After cooling the solution to 10 °C, 90 g of EDC was added, and the reaction mixture was stirred for another 30 min. Then, gaseous NH_3 was bubbled through the reaction mixture for 2 h. Next, the reaction solution was stirred for 16 h at ambient temperature. The solvent was removed in vacuo. The resulting residue was poured into H_2O and extracted with EtOAc. The organic phase was washed with aqueous 2 M $NaHCO_3$ and H_2O and dried, and the solvent was removed in vacuo. This residue (49 g) was suspended in 2-propanol. 2-Propanolic HCl was added, and the suspension was stirred for several hours at ambient temperature. The precipitate was collected and dried to give 35.5 g of a single diastereomer **11a** as a hydrochloride: mp 174−176 °C. ^{1}H NMR (D_2O) δ 0.9 (t, 3H), 1.25−1.4 (m, 4H), 1.6−1.7 (m, 2H), 1.78 (m, 1H), 3.63 (m, 1H), 4.43 (d, 1H). MS *m/z*: 160 (M^+). Anal. ($C_7H_{16}N_2O_2 \cdot 0.25\ HCl \cdot 0.5H_2O$) C, H, Cl, N.

Representative Procedure for the Preparation of Esters 14. *E*-2-[2-(Naphth-2-yl)ethen-1-yl]benzoic acid ethyl ester (14a): 25.5 g (0.17mol) of 2-vinylnaphthalene, 29.7 g (0.13mol) of 2-bromobenzoic acid ethyl ester (**12a**), 22.5 mL (0.16mol) of Et_3N, 0.54 g of $Pd(OAc)_2$, and 1.44 g of tri-*o*-tolylphosphine were added to 200 mL of DMF. After further adding 3 mL of H_2O, the reaction mixture was stirred at 100 °C for 3 h. Next, the mixture was poured into H_2O, and the aqueous solution was extracted with EtOAc. The EtOAc phase was washed several times with H_2O and dried, and the solvent was removed in vacuo. The residue was purified chromatographically on silica gel (eluant: heptane/EtOAc = 5/1) to afford 29.3 g (59%) of **14a** as oil. ^{1}H NMR (DMSO-d_6) δ 1.35 (t, 3H), 4.38 (q, 2H), 7.38 (d, J = 16 Hz, 1H), 7.4−8.2 (m, 12H). MS *m/z*: 302 (M^+).

2-[*E*-2-(4-Dimethylaminomethylphenyl)ethen-1-yl]benzoic acid methyl ester (14b): yield 74%. ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 6H) 3.86 (s, 3H), 7.19 (d, 1H) 7.30 (d, 2H) 7.40 (t, 1H), 7.53 (d, 2H), 7.62 (t, 1H) 7.80−7.90 (m, 2H). MS *m/z* = 309 (M^+).

2-[*E*-2-(4-Dimethylaminomethylphenyl)ethen-1-yl]-nicotinic acid ethyl ester dihydrochloride (14c): yield 42%. Mp 197−198 °C. ^{1}H NMR (DMSO-d_6) δ 1.37 (t, 3H), 2.60 (d, 6H), 4.30 (d, 2H), 4.38 (q, 2H), 7.63 (dd, 1H), 7.66 (d, 2H), 7.73 (d, 2H), 7.93 (d, J = 16 Hz, 1H), 8.20 (d, J = 16 Hz, 1H), 8.32 (d, 1H), 8.81 (d, 1H), 11.2 (broad, 1H, N^+H). MS *m/z*: 310 (M^+).

2-[*E*-2-(4-Diethylaminomethylphenyl)ethen-1-yl]benzoic acid ethyl ester (14d): yield 100%. ^{1}H NMR (DMSO-d_6) δ 0.97 (t, 6h), 1.35 (t, 3H), 2.45 (q, 4H), 3.53(s, 2H), 4.37 (q, 2H), 7.17 (d, J = 16 Hz, 1H), 7.33 (d, 2H), 7.40 (t, 1H), 7.50 (d, 2H), 7.60 (t, 1H), 7.80 (d, J = 16 Hz, 1H), 7.85 (t, 2H). MS *m/z*: 337 (M^+).

Representative Procedure for the Preparation of Carboxylates 15. *E*-2-[2-(Naphth-2-yl)ethen-1-yl]benzoic acid (15a): 29 g (95.9 mmol) of **14a** was dissolved in 200 mL of THF, and a solution of 10.8 g (192 mmol) of KOH in 150 mL of H_2O was added. The reaction mixture was refluxed for 12 h. Then the organic solvent was removed in vacuo. The resulting aqueous solution was diluted by adding 200 mL of H_2O and filtered. This solution was cooled on ice and acidified by adding 2 M HCl. The resulting precipitate was collected and dried to obtain 26.6 g (100%) of **15a**. Mp 211−212 °C. ^{1}H NMR (DMSO-d_6) δ 7.36 (d, J = 16 Hz, 1H), 7.4−7.65(m,4H), 7.8 (dd, 1H), 7.85−8.05(m,4H), 8.12 (d, J = 16 Hz, 1H). MS *m/z*: 274 (M^+).

2-[*E*-2-(4-Dimethylaminomethylphenyl)ethen-1-yl]benzoic acid (15b): yield 98%. Mp 215−216 °C. ^{1}H NMR (DMSO-d_6) δ 2.23 (s, 6H) 3.53 (s, 2H) 7.13 (d, J = 15 Hz, 1H), 7.35(m,3H) 7.55(m,3H), 7.80 (d, 2H), 7.95 (d, J = 15 Hz, 1H). MS *m/z*: 281 (M^+).

2-[*E*-2-(4-Dimethylaminomethylphenyl)ethen-1-yl]-nicotinic acid (15c): yield 76%. Mp 180−181 °C. ^{1}H NMR (DMSO-d_6) δ 2.45 (s, 6H), 2.92 (s, 2H), 7.30(m, 1H), 7.55 (d, 2H), 7.65 (d, 2H), 7.82 (d, J = 16 Hz, 1H), 8.10 (dd, 1H), 8.25 (d, J = 16 Hz, 1H), 8.60(m, 1H). MS *m/z*: 282 (M^+).

2-[*E*-2-(4-Diethylaminomethylphenyl)ethen-1-yl]benzoic acid (15d): yield 87%. Mp 180−181 °C. ^{1}H NMR (DMSO-d_6) δ 1.0 (t, 3H), 2.53 (q, 4H), 3.61 (s, 2H), 7.13 (d, J = 17 Hz, 1H), 7.36 (m, 3H), 7.52 (m, 3H), 7.8 (d, 2H), 7.92 (d, J = 17 Hz, 1H). MS *m/z*: 309 (M^+).

***N*-(1-Benzyl-2-carbamoyl-2-hydroxyethyl)-2-[*E*-2-(naphth-2-yl)ethen-1-yl]benzamide (16a)**: 1.52 g (5.6 mmol) of **15a**, 1.54 g (5.5 mmol) of 3-amino-2-hydroxy-3-phenylbutanoic acid amide (**11b**),[27] and 2.8 g (27.8 mmol) of Et_3N were dissolved in 200 mL of CH_2Cl_2/DMF (3/1) and stirred at ambient temperature for 30 min. HOBt (0.75 g, 5.6 mmol) was added, and the reaction solution was cooled to 0 °C. Then, 1.1 g (5.6 mmol) of EDC was added, and the reaction mixture was stirred for another 60 min at 0 °C. Next, the solution was stirred for 16 h at ambient temperature. The reaction solution was concentrated in vacuo, and the resulting residue was redissolved in 500 mL of H_2O. The aqueous solution was rendered alkaline by adding 2 M $NaHCO_3$ solution, which caused the precipitation of a white solid. The solid was collected and dried to give 1.86 g (74%) of **16a**. Mp 232−234 °C. ^{1}H NMR (DMSO-d_6) δ 2.84 (dd, 1H), 2.96 (dd, 1H), 3.99 (dd, 1H), 4.57 (m, 1H), 5.76 (d, 1H, OH), 7.17 (m, 1H), 7.25−7.36 (m, 8H), 7.42−7.55 (m, 4H), 7.63 (dd, 1H), 7.86 (d, 1H), 7.89−7.95 (m, 4H), 8.2 (d, 1H). MS *m/z*: 448 (M^+).

***N*-(1-Carbamoyl-1-hydroxyhex-1-yl)-2-[*E*-2-(4-dimethylaminomethylphenyl)ethen-1-yl]benzamide (16b)**: 11.3 g (40.2 mmol) of **11a** and 12.1 g (120 mmol) of Et_3N were dissolved in 300 mL of dry DMF. Then, 5.4 g (39.2 mmol) of HOBt was added. After the reaction mixture was stirred for 15 min, 7.9 g (40.2 mmol) of **11a** was added, and stirring was

continued for 30 min. The reaction mixture was cooled to 10 °C, and 8.4 g (43.8 mmol) of EDC was added portionwise to the solution. Next, the solution was stirred at ambient temperature for 16 h. Then, the reaction solution was poured into 450 mL of a aqueous 4 M NaCl solution, and the resulting precipitate was collected, washed with H_2O and ether, and finally dried to afford 14.3 g (84%) of **16b**. Mp 195−196 °C. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3H) 1.1−1.4 (m, 4H) 1.5−1.7 (m, 2H) 2.15 (s, 6H) 3.35 (s, 2H) 4.0 (m, 1H) 4.3 (m, 1H) 5.7 (d, 1H, OH) 7.15−7.50 (m, 11H) 7.85 (m, 1H) 8.05 (d, 1H). MS *m/z*: 485 (M^+). Anal. ($C_{25}H_{33}N_3O_3 \cdot 0.75H_2O$) C, H, N.

***N*-(1-Benzyl-2-carbamoyl-2-hydroxyethyl)-2-[*E*-2-(4-dimethylaminomethylphenyl)ethen-1-yl]nicotinamide (16c):** 12.5 g (44.3 mmol) of **15c**, 10 g (44.3 mmol) of 3-amino-2-hydroxy-3-phenylbutanoic acid amide (**11b**),[28] 2 g (14.8 mmol) of HOBt, and 21.5 mL (155 mmol) of Et_3N were added successively to 500 mL of DMF and stirred at ambient temperature for 30 min. EDC (7.7 g, 44.3 mmol) was added in portions, and the reaction mixture was stirred for 16 h. Then the solvent was removed in vacuo. After suspending the residue in 1000 mL of H_2O, the aqueous phase was made alkaline by adding 45 mL of 2 M NaOH. The solution was cooled on ice, and the resulting precipitate was collected, washed with H_2O, dried, and crystallized from EtOH to yield 14.7 g (73%) of **16c**: Mp 212−213 °C. ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 6H), 2.79 (d, 1H), 2.80 (s, 1H), 3.41 (s, 2H), 4.11 (dd, 1H), 4.58 (m, 1H), 5.98 (d, OH), 7.07 (d, J = 15.7 Hz, 1H), 7.12 (m, 1H), 7.2−7.45 (m, 11H), 7.60 (dd, 1H), 7.73 (d, J = 15.7 Hz, 1H), 8.48 (d, 1H), 8.59 (dd, 1H). MS *m/z*: 458 (M^+). Anal. ($C_{29}H_{34}N_4O_3$) C, H, N.

***N*-(1-Benzyl-2-carbamoyl-2-hydroxyethyl)-2-[*E*-2-(4-diethylaminomethylphenyl)ethen-1-yl]benzamide (16d):** 20 g (64.6 mmol) of **15d**, 15 g (64.6 mmol) of 3-amino-2-hydroxy-3-phenylbutanoic acid amide (**11b**),[27] and 2.9 g (21.5 mmol) of HOBt were added to 1400 mL of dry DMF. Et_3N (32 mL, 226 mmol) was added, and the resulting solution was cooled to 0 °C. Then 12.4 g (64.6 mmol) of EDC was added, and the solution was stirred at ambient temperature for 16 h. The solvent was removed under reduced pressure which afforded an oily residue. This oil was redissolved in EtOAc. This solution was subsequently washed with 2 M $NaHCO_3$ and H_2O and dried, and the solvent was removed in vacuo. The resulting residue was dissolved in CH_2Cl_2/MeOH, and the subsequent careful addition of *n*-pentane caused a precipitation of a white solid which was collected by filtration to afford 22.5 g (72%) of **16d**. Mp 195−196 °C. ^{1}H NMR (DMSO-d_6) δ 0.98 (t, 6H), 2.45 (q, 4H), 2.80 (m, 2H), 3.53 (s, 2H), 4.10 (m, 1H), 4.58 (m, 1H), 5.93 (d, 1H, OH), 7.0−7.5 (m,16H), 7.78 (d, 1H), 7.23 (d, 1H). MS *m/z* = 485 (M^+). Anal. ($C_{30}H_{35}N_3O_3 \cdot 0.5H_2O$) C, H, N.

***N*-(1-Benzyl-2-carbamoyl-2-oxoethyl)-2-[*E*-2-(naphth-2-yl)ethen-1-yl]benzamide (5a):** 1.83 g (4.1 mmol) of **16a** and 4.11 g (50.8 mmol) of Et_3N were dissolved in 75 mL of dry DMSO. Py·SO_3 (6.5 g, 40.6 mmol) was added, and the resulting reaction mixture was stirred for 16 h at ambient temperature. The reaction solution was poured into 500 mL of H_2O, and the aqueous solution was extracted twice with EtOAc. The combined EtOAc phases were washed with H_2O and dried, and the solvent was removed in vacuo. The residue was crystallized from EtOAc/*n*-pentane to afford 1.66 g (71%) of **5a** as a white solid. Mp 155−159 °C. ^{1}H NMR (DMSO-d_6) δ 2.9 (dd, 1H), 3.26 (dd, 1H), 5.45 (m, 1H), 7.10−7.60 (m,13H), 7.73 (d, 1H), 7.8−8.0 (m, 5H), 8.23 (s, 1H), 8.98 (d, 1H). MS *m/z*: (448, M^+). Anal. ($C_{25}H_{27}N_3O_3 \cdot 0.5H_2O$) C, H, N.

***N*-(1-Carbamoyl-1-oxohex-1-yl)-2-[*E*-2-(4-dimethylaminomethylphenyl)ethen-1-yl]benzamide (5b):** 9.7 g (22.9 mmol) of **16b** and 43.9 g (229 mmol) of EDC were dissolved in 400 mL of DMSO. At ambient temperature, 11.8 g (91.5 mmol) of dichloroacetic acid was dropped into the stirred solution. The resulting reaction mixture was stirred for further 30 min at ambient temperature. Then the reaction mixture was poured into 1200 mL of H_2O, and the pH of this aqueous solution was adjusted to 8 by adding 2 M $NaHCO_3$. The resulting solution was extracted with EtOAc, and this organic phase was separated and immediately acidified by adding 2-propanolic HCl. The solvent was removed in vacuo, and the resulting solid was crystallized from acetone/EtOH (4:1) to obtain 5.5 g (52%) of the hydrochloride of **5b**. Mp 132−134 °C. ^{1}H NMR (DMSO-d_6) δ 0.84 (t, 3H), 1.30 (m, 2H), 1.39 (m, 2H), 1.58 (m, 1H), 1.81 (m, 1H), 2.66 (s, 6H), 4.22 (s, 2H), 5,17 (ddd, 1H), 7.30 (d, J = 16.4 Hz, 1H), 7.38 (dd, 1H), 7.39 (d, 1H), 7.48 (d, J = 16.4 Hz, 1H), 7.49 (t, 1H), 7.56 (d, 2H), 7.63 (d, 2H), 7.83 (broad, 1H, NH), 7.88 (d, 1H), 8.12 (broad, 1H, NH), 8.4 (broad, 1H, NH), 10.7 (broad, 1H, NH). MS *m/z*: 421 (M^+). Anal. ($C_{25}H_{31}N_3O_3 \cdot HCl \cdot H_2O$) C, H, Cl, N.

***N*-(1-Benzyl-2-carbamoyl-2-oxoethyl)-2-[*E*-2-(4-diethylaminomethylphenyl)ethen-1-yl]nicotinamide (5c):** 2.0 g (4.36 mmol) of **15c** and 9.0 g (43.6 mmol) of DCC were dissolved in 40 mL of CH_2Cl_2/DMSO (2/1). After the solution was cooled to 10 °C, 1.44 mL (17.45 mmol) of $Cl_2CHCOOH$ was dropped into the reaction solution. Then the reaction mixture was stirred for 1 h at ambient temperature. The reaction solution was poured into ice and water, and the resulting precipitate was filtered off. The resulting aqueous solution was diluted with 400 mL of H_2O and washed several times with EtOAc. The pH of the aqueous phase was adjusted to pH 8 by adding 2 M $NaHCO_3$. This aqueous phase was extracted several times with EtOAc. The combined EtOAc phases were washed with H_2O and brine and dried, and the solvent was removed in vacuo. The resulting solid was suspended in 200 mL of water and redissolved by adding 5.7 mL of 2 M HCl. The solution was filtered, and the filtrate was frozen and freeze-dried in vacuo to give a solid. This solid was recrystallized from ethanol to afford 1.5 g (69%) of **5c** as a dihydrochloride. Mp 189−190 °C. ^{1}H NMR (DMSO-d_6) δ 2.71 (d, 6H), 2.87 (dd, 1H), 3.27 (dd,1H), 4.32 (d,4H). 5.47 (m, 1H), 7.25 (t, 1H), 7.27−7.36 (m,6H), 7.47 (dd,1H), 7.63 (dd, 4H), 7.70 (d, J = 15 Hz, 1H), 7.87 (d,1H), 7.92(broad,1H, NH), 8.26 (broad,1H,NH), 8.68 (dd, 1H), 9.25 (d,1H), 10.9(broad, 1H). ^{13}C NMR (DMSO-d_6) δ 34.6 (t), 41.4, 56.0, 58.7, 121.4, 123.4, 126.5, 127.7, 128.2, 128.9, 131.5, 131.6, 132.2 136.1, 136.6, 137.4, 139.9, 146.5, 148.7, 162.7, 165.8, 196.3. MS *m/z*: 474 (M^+ + H_2O), 456 (M^+). Anal. ($C_{29}H_{32}N_4O_3 \cdot 2HCl \cdot 3H_2O$) C, H, Cl, N.

***N*-(1-Benzyl-2-carbamoyl-2-oxoethyl)-2-[*E*-2-(4-diethylaminomethylphenyl)ethen-1-yl]benzamide (5d):** A solution of 30 g (61 mmol) of **16d** in 110 mL of DMSO and 127.2 g (61 mmol) of DCC in 330 mL of CH_2Cl_2 were combined. At 15 °C, 31.9 g (150 mmol) of dichloroacetic acid was added, and stirring was continued for 1 h. Then the reaction mixture was filtered, and the filtrate was poured into 1.8 L of ice/water. The aqueous phase was extracted three times with EtOAc, and these organic phases were discarded. The pH of the resulting aqueous phase was adjusted to 8 by adding 2 M $NaHCO_3$. Then the aqueous phase was again extracted with EtOAc. The organic phase was washed with H_2O and brine and dried, and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2/acetone, and 4.6 g of methanesulfonic acid was added. Ether was carefully added to cause precipitation of a solid which was collected and dried to obtain 21.8 g (61%) of the methanesulfonate of **5d**. Mp 109−111 °C. ^{1}H NMR (DMSO-d_6) δ 1.23 (t, 3H), 2,34 (s, 3H), 2.86 (dd, 1H), 3.23 (dd, 1H), 3.08 (q, 4H), 4.29 (s, 2H), 5.42 (m, 1H), 7.1−7.6 (m, 9H), 7.4−7.7(6H), 7.85 (d, 1H), 7.92 (s, 1H), 8.20 (s, 1H), 8.97 (d, 1H). ^{13}C NMR (DMSO-d_6): δ = 8.5, 34.5, 39.3, 46.0, 54.7, 55,8, 123.5, 124.5, 125.1, 126.3, 126.6, 126.8, 127.3, 127.5, 128.0, 128,1, 128.4, 128.9, 129.5, 129.7, 130.3, 131.2, 132.7, 135.7, 137,8, 139.0, 163.0, 168.7, 196.8. MS *m/z*: 501 (M^+ + H_2O), 483 (M^+). Anal. ($C_{30}H_{33}N_3O_3 \cdot 1.25CH_3SO_3H$) C, H, N, S.

Calpain I Inhibition. Calpain I was purified from human erythrocytes according to the method of Gabrijelcic-Geiger et al.[42] In brief, after four chromatographic steps (DEAE-Sepharose, Phenyl-Sepharose, Superdex 200, and Blue-Sepharose), the enzyme was obtained at a purity of >95%, as assessed by SDS−PAGE and HPLC analysis. Its composition was determined by endoproteinase Lys-C cleavage, peptide mapping, and protein sequencing. The buffer solution used in experiments contained 50 mM Tris-HCl, pH 7.5; 0.1 M NaCl; 1 mM dithiothreithol; 0.11 mM $CaCl_2$. Suc-Leu-Tyr-AMC

(Bachem/Switzerland) was used as fluorogenic calpain substrate.[27,28,43] Fluorescence of the cleavage product 7-amino-4-methylcoumarin (AMC) was measured continuously in a Spex-Fluorolog fluorimeter at λ_{ex} = 380 nm and λ_{em} = 460 nm. The fluorescence signal was linear over a time period of 60 min.

The inhibitors and the calpain substrate were dissolved in DMSO, but the DMSO concentration in the final experimental solutions did not exceed 2%.

In a typical experiment, 10 μL of substrate (250μM final concentration) and then 10 μL of μ-calpain (μg/mL final concentration, i.e., 18 nM) were added to 1 mL of buffer solution. After 15 min, 10 μL of the inhibitor solution (50 or 100 μM solution in DMSO, i.e., 500 or 1000 nM final concentration) were added, and the experiment was continued for 40 min. All this time, fluorescence was recorded over the time. K_i values were determined using the classical equation for reversible inhibition, under steady-state conditions and calpain concentrations much smaller than inhibitor concentrations, i.e., $K_i = I/(v_0/v_i) - 1$; where I = inhibitor concentration, v_0 = initial velocity before adding the inhibitor; v_i = reaction velocity at equilibrium. A study was done to compare the autocatalytic effect at different temperatures and calcium concentrations. The decrease in rate (autocatalysis) over time is virtually absent at 12 °C and 0.11 mM Ca^{2+} (data not shown) and all studies were therefore conducted at these conditions.

Calpain-Mediated Degradation of Tyrosine Kinase pp60src in Platelets. The method is described more in detail by Oda et al.[35] Fresh human, citrate-treated blood was centrifuged at 200g for 15 min to obtain the platelet-rich plasma which was pooled and subsequently diluted with the same volume of a buffer (68 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.24 mM $NaH_2PO_4 \cdot H_2O$, 12 mM $NaHCO_3$, 5.6 mM glucose, 1 mM EDTA, pH 7.4). After centrifugation and washing with the buffer, the pellet was diluted with buffer to adjust the platelet count to 10^7 cells/mL. The experiments were carried out at 37 °C.

In a typical experiment, 5×10^6 platelets were preincubated with the inhibitor (as DMSO solution) for 5 min. 1 μM ionophore A23187 (Calbiochem) and 5 mM $CaCl_2$ were added to activate the platelet calpain. After 5 min, the platelets were briefly centrifuged at 13000 rpm, and the pellet was taken up in SDS sample buffer (20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/mL leupeptin, 10 μM pepstatin, 10% glycerol, and 1% SDS). The proteins were fractionated on a 12% gel. To examine pp60src and its 52 kDa and 47 kDa cleavage products, proteins were transferred electrophoretically from the gel onto a nitrocellulose membrane and identified by Western blotting. The polyclonal rabbit anti-Cys-src (pp60src) antibody employed was obtained from Biomol-Feinchemikalien (Hamburg). This primary antibody was detected by using a goat HRP-coupled second antibody (Roche, Germany). Cleavage of pp60src was quantified densitometrically. Nonactivated platelets (control 1: no cleavage) and platelets treated with ionophore and calcium (control 2: corresponds to 100% cleavage) were used as controls. The ED_{50} values were determined as the concentration of inhibitor at which the intensity of the color reaction of the 60 kDa band was 50% of the mean of intensities of control 1 minus control 2.

Traumatic Brain Injury in the Rat. A group of male Sprague Dawley rats (180–280 g) were used and divided into a treated and a control group (each 20 animals). The rats were anaesthetized with halothane (1–2%, 4% for initial induction) in a mixture of 70% N_2O and 30% O_2 delivered through a loose fitting face mask. The right temporo-parietal region of the head was exposed, a small craniotomy was made, and a male Luer-Lock fitting was fixed over the opening using dental cement. After hardening of the cement, the rat was attached to a fluid percussion device. The device consisted of a saline-filled cylinder, one end of which was connected, via a pressure transducer, to a female Luer-Lock fitting which was connected to the rat's skull, thus creating an airtight fluid-filled chamber. The other end of the cylinder was stoppered with a piston. A heavy metal pendulum was dropped onto the piston from a predetermined height to create a burst of fluid pressure on the surface of the rat's brain (2.5–2.9 bar). Fourteen days later, the rat was killed and the brain removed. After cryoprotection, the brain was cut to 30 μm slices (coronal slices) on a freezing microtome. After Toluidine Blue staining, the extent of injury was measured by counting the hilar neurons of the dentate gyrus, both on the side of the injury and the contralateral side.

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